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(54) Title: DERMAL SHEATH TISSUE IN WOUND HEALING

(57) Abstract

The invention concerns the use of dermal sheath tissue and/or cells derived therefrom in wound healing systems. Specifically, the relevant features of this tissue type have been exploited to provide a novel wound healing material that has application in the provision of new therapeutic compositions and new surgical dressings where wound closure and minimal scarring is desirable.

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DERMAL SHEATH TISSUE IN WOUND HEALING

The invention relates to the use of dermal sheath tissue and/or cells derived

5   therefrom and/or portions of hair follicles containing these and other cell
populations for use particularly, but not exclusively in wound healing and/or
skin models.

Skin is a highly complex organ covering the external surface of the body and

10   merging, at various body openings, with the mucous membranes of the
alimentary and other canals. It has multiple functions such as preventing water
loss from the body, but predominantly acts as a protective barrier against the
action of physical, chemical and bacterial agents on deeper tissues. Skin is
elastic and except for a few areas such as the palms, soles and ears it is loosely

15   attached to underlying tissue. It varies in thickness from 0.5mm (0.02 inches)
on the eyelids to 4mm (0.17 inches) or more on the palms and soles.

Skin is composed of two layers (please refer to Figure 1 which illustrates an
anatomical cross-sectional view through a slice of skin), the outer layer, which

20   is comparatively thin (0.1 mm) is called the epidermis, or cuticle, it is several
cells thick and has an external, horny layer of dead cells that are constantly shed
from the surface and replaced from below by a basal layer of cells, called the
stratum germinativum.   The epidermis is composed predominantly of
keratinocytes which make up over 95% of the cell population, the rest include

25   dendritic cells such as Langerhans cells and melanocytes. It is essentially
cellular and non-vascular, there being relatively little extracellular matrix except
for the layer of collagen and other proteins beneath the basal layer of

keratinocytes. Keratinocytes of the basal layer are constantly dividing, and daughter cells subsequently move outwards, where they undergo a period of differentiation and are eventually sloughed off from the surface. The inner layer

5   of the skin is called the dermis and is composed of a network of collagenous extracellular material, elastic fibres, blood vessels and nerves. Contained within it are hair follicles with associated sebaceous glands (collectively known as the pilosebaceous unit) and sweat glands. The interface between the epidermis and dermis is extremely irregular and consists of a succession of papillae, or finger

10   like projections. Beneath the basal epidermal cells along this interface the specialised extracellular matrix is organised into a distinct structure called the basement membrane.

The mammalian hair fibre is the product of a small peg of tissue known as the

15   hair follicle which lies immediately underneath the skin's surface. The distal part of said follicle is in direct continuation with the cutaneous epidermis externally. Although small, the follicle comprises a highly organised system of recognisably different layers arranged in concentric series. Active hair follicles extend down through the dermis, the hypodermis (a loose layer of connective

20   tissue), and the fat or adipose layer.

At the base of any active follicle lies the hair bulb, which consists of a body of dermal cells, known as the dermal papilla, contained in an inverted cup of epidermal cells known as the epidermal matrix (please refer to Figure 1).

25   Irrespective of follicle type, the hair fibre, together with several supportive epidermal layers, is produced by germinative epidermal cells at the very base of this epidermal matrix. The lowermost dermal sheath is contiguous with the papilla basal stalk, from where it curves externally around all of the epidermal

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layers of hair matrix as a thin covering of tissue, and then continues as a tube or sleeve for the length of the follicle. The dermal sheath is otherwise known as the connective tissue sheath.

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Developing skin appendages such as feather and hair follicles rely on interaction between the skin's two layers, the epidermis and the dermis. In embryonic development, a sequential exchange of information between these layers underpins a complex series of morphogenetic processes culminating in the

10   formation of adult follicle structures. However, following maturity, and in contrast to general skin dermal and epidermal cells, certain hair follicle cell populations retain embryonic-type inductive, interactive and biosynthetic behaviours. These properties are likely to derive from the very dynamic nature of the cyclically productive follicle, in which repeated tissue remodelling

15   necessitates a high level of dermal-epidermal interactive communication, as is vital for embryonic development and, as would be desirable in any form of tissue reconstruction.

Hair fibre is produced at the base of an active follicle at a very rapid rate

20   (0.4mm per day in the human scalp follicles and up to 1.5mm per day in the rat vibrissa or whiskers), which means that cell proliferation in the follicle epidermis ranks amongst the fastest in adult tissues (1).

The most dynamic region of the hair follicle is the deeply embedded end bulb,

25   where local dermal-epidermal interactions drive active fibre growth. This same region is also central to the developmental changes and tissue remodelling involved in the hair fibre's or appendages precise alternation between growth and regression phases. As a key player in the activities, the dermal papilla

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appears to orchestrate the complex program of differentiation that characterises hair fibre formation from the primitive germinative epidermal cell source (2-5).
The lowermost dermal sheath initiates below the papilla basal stalk, from where

5   it curves outwards and upwards to externally enclose all of the layers of the epidermal hair matrix as a thin cup of tissue. (Please refer to Figure 1). The dermal sheath continues as a tubular arrangement for the length of the follicle, as does the epidermal outer root sheath which lies immediately internal to it in between the two layers is a specialised basement membrane termed the glassy

10   membrane. The outer root sheath constitutes little more than an epidermal monolayer in the lower follicle, but becomes increasingly thickened more superficially.

Pioneering studies established that rat whisker follicles whose end bulbs had

15   been amputated, could regenerate all of the crucial elements that were required to restore fibre growth (6). They also revealed that the isolated dermal papilla had particularly powerful interactive capabilities in being able to induce completely new hair follicles when re-implanted in vivo (2). Subsequent experiments gave further support to what had been found in rats (4, 7-9), and

20   confirmed that papillae from other species, including mice (10, 11) and sheep (12) had similar interactive capabilities. Human facial (13) and axillary (14) follicles in situ, as well as, isolated human follicles transplanted to rodent hosts (15), have also been reported to regenerate following amputation. Further studies in vitro strongly suggested that human follicle tissues and cells displayed

25   the highly specialised interactive properties that were seen in their rodent counterparts (16).

Whilst the individual anatomical components and cell sub-populations of skin

are well established their intra/inter biochemical interactions and control mechanism remains largely a matter for speculation and intense research.

5   Destruction of extensive areas of skin is caused by injury, burns, disease or large ulcers. Regeneration of skin over denuded areas takes place naturally by cell proliferation from the surrounding margins of healthy undamaged skin and the underlying skin appendages when these remain. A specific clinical problem that occurs naturally as a result of wound healing is hypertrophic scarring,

10   where the balance between new collagen production and collagen breakdown is not in equilibrium, and there is an overproduction of scar tissue which can inhibit coverage of the wound with new epidermis. This is quite common, but it usually subsides. However in some cases the condition can worsen and keloid scars (masses of extracellular matrix that keep on growing) can be produced.

15   Clinically this can be treated by steroids if minor, but more serious cases are extremely difficult to treat and often result in surgery. However, if the area of denuded/damaged skin is large and of full thickness then complete coverage of the damaged area is facilitated by the use of skin grafts, sections of skin of either full or partial thickness *are* removed from a remote part of the body

20   (donor site) and applied to the raw surface (recipient site).

It should be noted that generally grafts are taken from skin transplanted from the body of the same individual (autographs). In a successful transplant the graft is nourished at first by the serum oozing from the damaged area and

25   subsequently by capillary invasion from underlying tissue into the graft.

Graft survival has long been acknowledged to be affected by its immunogenicity in relation to the site it is transplanted to. As an extreme

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example, females are very intolerant of male tissue - largely due to their reaction against male-specific (H-Y) antigens (17). The skin is well known for its notoriously poor support or tolerance in this context (18, 19).

5   Over the years a multitude of approaches have been followed in an attempt to create a viable model with which to study cutaneous physiology and morphology, and/or which can be used for medical grafting procedures.

There are four or more common types of dermal support in skin wound models:

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a)   native collagen (dermis treated to remove cellular elements which subsequently becomes re-cellularised);

b)   collagenous gels or lattices (made from extracted collagen which is then
15   reconstituted) and,

c)   highly complex mixtures of reconstructed collagen and a multitude of extracellular matrix products (from sources as diverse as mouse tumours and shark fins).

20

d)   neonatal or young donor skin fibroblasts (thus less immunogenic) grown on a biomatrix, and allowed to attach and produce their own extracellular matrix. This is then transplanted in either the cellular form as a "living skin" or cell free, using the extracellular matrix that has been produced
25   by the cells.

All of these methodologies incorporate the use of ordinary skin fibroblasts, and none produce models or substitutes that are truly representative of normal skin

structure. Moreover, they are often very time consuming and extremely expensive to prepare.

5   Wound healing or contracture can be a painful process both physiologically and psychologically. Scarring can lead to disfiguration and deformation, in some cases the taughtness and/or adhesions, as a result of scar tissue formations, can cause damage to surrounding and/or underlying tissue and/or loss of regional elasticity and/or sensation. In humans, much of the problem associated with full

10   thickness wounds/burns/injury/trauma is that a normal thickness dermis is not restored, which can result in depressions and/or indentations in the scar region. Many animals have a "looser" skin than humans and have a greater capacity for contraction, as exemplified by removal of a large 2cm full thickness patch of skin from a rabbit back and reduction of the site to a scar, that is a barely visible

15   line, in a matter of two weeks. Consequently aiding/enhancing contraction could reduce the size of scarred region. Furthermore, it is our belief that the known phenomenon of rapid human scalp wound healing, compared to wound healing rates in other parts of the body which have lower follicle density, or with fewer large active follicles, is due to the abundance of available dermal

20   sheath cells which, as myofibroblasts, assist in wound contraction. This idea is against the prevailing wisdom, in which the rapid healing properties of the scalp skin are attributed to greater vasculature in this region.

Wound contracture is a very important part of the process by which skin heals,

25   and it is thought that a transient population of cells called myofibroblast, effect this closing of the damaged site by contraction. The exact source of the myofibroblast has not been identified, but said cells are characterised by their expression of alpha smooth muscle actin. An invention to improve wound

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healing would have immediate benefit and wide application since it is estimated that over 2 million people suffer serious burns in the US alone each year, and many of those require grafts.

5   Several experimental approaches to artificial skin grafts have been tried, for example epithelial sheets have been grown in culture from interfollicular skin and grafted back onto the donor at a later date - but although life-preserving, this method is often unsatisfactory because the sheets are delicate and difficult to transfer.   Also, since there is no supportive dermis involved, dermal

10   indentations are left at the graft site because a normal full thickness dermis is not restored. Their simplicity leaves them unrepresentative of a skin model for studies *in vitro*, while in relation to grafting, at best, they merely act as covers while the patients own skin can recover.

15   Alternative approaches have been attempted employing skin basal and outer root sheath epidermal cells have been cultured on collagen gels (with or without skin fibroblasts), or other natural or synthetic dermal substitutes, with the same ultimate objective, but again may of these approaches have specific limitations, usually related to survival or inability to resemble normal skin. Of the prior art

20   approaches it is claimed that outer root epidermal sheath cells in combination with a dermal component provide the best results on wound patients. Thus, in spite of demand and the provision of complex supportive biochemical constituents, the current *in vitro* skin models are deficient in that:

25   1)   their epidermal component does not undergo normal differentiation;

     2)   they do not develop the skin's normal biosynthetic dermal-epidermal barrier (a kind of biological filter and message processor) called the

basement membrane;

3)   they lack constituents such as hair follicles and sweat glands;

4)   they are fragile, difficult to handle and cannot be maintained;

5)   they cannot avoid disfigurement because they cannot fill tissue void;

6)   they do not prevent infection or restore living/effective tissue so inflexible non-functional scars can develop.

Therefore in terms of faithful *in vitro* systems, and as material for skin replacement, present models/substitutes are deficient.

Recent papers in the area emphasise the desperate need for more research to try and find a suitable backing material for epidermal grafts that will promote a high level of 'take'. We propose that the prior art failures are largely due to the lack of a favourable wound bed dermis for epidermal cells to attach to, when the full thickness of skin has been lost - we believe that the dermal sheath cells are the natural candidates for this role, and that successful tissue engineering can only occur when the appropriate cells for the job are employed.

The most important of all cells types are those at the source of every biological system ie stem cells, since they vitally sustain and replenish the more differentiated descendent population and as they become specialised develop a characteristic function. Yet these are the cells which are least understood in terms of their distribution, behaviour and the factors by which they may be

defined. The ability to provide significant numbers of pure, unstimulated, undifferentiated, primitive stem cells from an adult organ would be likely to have a broad impact on our fundamental understanding of cell biology, and

5   would yield positive and promising approaches to future therapeutic advances.

Serendipitously, we have studied hair follicles and identified a specific cell population with immune privilege and stem specific cell potential that can be used in a most advantageous way to aid and enhance wound healing.

10

We have found that simple forms of follicle cell(s) provide dermal-epidermal recombination (under very basic culture conditions) resulting in previously undocumented basement membrane formation and appendage-specific epidermal differentiation, and that these follicle cells can readily exhibit certain

15   major characteristics that to date have been missing from skin model systems. Further natural attributes that pre-dispose follicle cells as candidates for the applications of wound healing include their: similarity to wound myofibroblasts; exhibition of stem cell-type qualities; production of a unique embryonic-type extracellular matrix and, the fact that they exhibit impressive regenerative and

20   inductive abilities.

Thus, in at least three key areas (basement membrane formation, normal epidermal differentiation over a naturally supportive dermis and the incorporation of skin appendages) we are able to deliver immediate

25   improvements to the currently available skin graft alternatives and *in vitro* skin models. Hence, by utilising a specific and isolated tissue or cell type ie dermal sheath tissue and/or cells derived therefrom and incorporating them in a reconstructed graft or graft composite, we are able to produce a more

appropriate skin substitute which overcomes many of the problems associated with the prior art.

5   It is therefore an object of the invention to provide a new wound healing system that employs self derived cells/tissues and/or their attributes.

It is a yet further object of the invention to provide a wound healing system that employs non-self derived cells tissues and/or their attributes.

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It is a yet further object of the invention to provide a wound healing system of multi-potential use ie for use in acute and/or chronic and/or minor and/or severe wound healing situations.

15   According to a first aspect of the invention there is provided dermal sheath tissue and/or cells derived therefrom for use in a wound healing system.

In a preferred embodiment of the invention said dermal sheath tissue and/or said cells is/are derived from a selected portion of a follicle ideally the lower third

20   thereof and even more ideally are derived from an ring of cells about or around said follicle, ideally an inner ring, and most ideally said tissue/cells is/are positive for smooth muscle actin.

In a preferred embodiment of the invention said tissue and said cells derived

25   therefrom are provided or combined with at least one other cell type from a hair follicle, and most ideally are combined with tissue comprising the dermal papilla, or cells derived therefrom. This combination is favoured because our experiments have shown that dermal papilla tissue, or cells derived therefrom

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may assist in the closure of wound and in the reduction of scar tissue.

In a yet preferred embodiment of the invention there is provided a wound
healing system comprising a suitable matrix material having incorporated and/or
embedded therein and/or associated therewith and/or attached thereto dermal
sheath tissue and/or cells derived therefrom. Ideally said matrix material
comprises native collagen or collagenous gels or lattices constructed from
reconstituted collagen or highly complex mixtures of reconstructed collagen and
a multitude of extracellular matrix products or any other suitable matrix material
known to those skilled in the art, the selection of which is not intended to limit
the scope of the invention.

In a yet further preferred embodiment of the invention there is provided a
surgical dressing comprising a web material and a suitable matrix material, at
least one of which materials has incorporated and/or embedded therein and/or
associated therewith and/or attached thereto dermal sheath tissue and/or cells
derived therefrom. Ideally said surgical dressing is conventional, the selection
of which is not intended to limit the scope of the invention.

In a yet further preferred embodiment of the invention there is provided a
therapeutic composition comprising a suitable carrier for dermal sheath tissue
and/or cells derived therefrom, ideally said carrier can be formulated to have
anti-bacterial properties and/or anti-septic properties and more ideally further
include growth promoting additives and/or local anaesthetics. Ideally said
therapeutic composition may be adapted to be applied topically in the form of
dermal sheath cells suspended in a suitable carrier solution/gel/cream/emollient;
alternatively said composition may be adapted to be administered by injection

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and so comprise a carrier solution; alternatively still, said carrier may be incorporated and/or embedded therein and/or associated therewith and/or attached thereto a plaster or bandage or the like.

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In a yet further preferred embodiment of the invention there is provided a suitable matrix material having incorporated and/or embedded therein and/or associated therewith and/or attached thereto dermal sheath tissue and/or cells derived therefrom for implantation.

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According to a second aspect of the invention there is provided dermal sheath tissue and/or cells derived therefrom for use in a reconstructed graft for use in the treatment of skin injury where dermal replacement is required.

15   In yet a preferred embodiment of the invention said tissue and/or cells are further provided or combined with at least one other cell type, said cell type being derived from a hair follicle and ideally comprising dermal papilla tissue and/or cells derived therefrom.

20   In a yet further preferred embodiment of the invention there is provided dermal sheath tissue and/or cells derived therefrom for use in in vitro modelling, ideally, and optionally, in combination with at least one other cell population derived from skin.

25   According to a third aspect of the invention there is provided a wound healing system as hereinbefore described for use in treatment of acute and/or chronic and/or minor and/or severe wound healing; and/or cartilage repair and/or bone repair and/or muscle repair and/or connective tissue repair and/or blood vessel

repair.

According to a fourth aspect of the invention there is provided dermal sheath

5   tissue and/or cells adapted so as to provide a protective layer of cells/capsule

wherein said layer of cells/capsule is suitably positioned on or about an organ

that is to be allografted to a patient/recipient. In this aspect of the invention the

immunoprotective quality of the dermal sheath tissue and/or cells derived

therefrom is exploited.

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In any of the above aspects of the invention said dermal sheath tissue may be

derived from self or more preferably from non-self.

In summary, we believe the dermal sheath tissue and/or cells derived therefrom

15   have an important part to play in wound healing and/or wound modelling

because this tissue and/or cells derived therefrom has been shown to participate

in wound contraction, wound dermis replacement-including the replacement of

a variety of dermis types depending upon the site of the wound, increasing

wound strength and status, supporting epidermal growth, a reduction in

20   scarring-indeed this tissue type, and/or in combination with dermal papilla cells

can lead to scarless wound healing, and moreover, this tissue type can be stored

long term at low temperatures and still retain the aforementioned properties,

thus wound healing therapeutics and wound cell models can be reliably

manufactured and then stored for future use. Additionally, this tissue and/or

25   cells derived therefrom can exist for a long time in culture under extreme stress,

thus wound healing therapeutics and/or wound cell models of this sort are

robust in nature, another favourable advantage in terms of storage, and

subsequent application.

An embodiment of the invention will now be described by way of example only with reference to the following Figures wherein:

5   Figure 1 represents a diagrammatic illustration of an anatomical cross-sectional view through a slide of skin.

Figure 2 represents a diagrammatic representation of procedures.

10   Figure 3 represents pictorial evidence of isolated dermal papilla (P) and sheath (S) tissue microdissected from male scalp hair follicle end bulbs: as shown in Figure 2e, marked by a star (*).

Figure 4 represents pictorial evidence of two hair fibres which have been
15   produced in the immediate vicinity of the male dermal sheath-implanted female skin wound protected by a small silicone rubber collar.

Figure 5 represents pictorial evidence of Figure 4 after the silicone collar (and plaster attachment) has been removed.
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Figure 6 represents pictorial evidence of a histological section through an end bulb region of an induced follicle, revealing an Alcian blue-positive stained papilla (P).

25   Figure 7 represents pictorial evidence of a lower portion of an induced follicle which can be seen to stain positively following in situ hybridisation with a Y-chromosome-specific DNA probe, realised via digoxygenin label.

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Figure 8 represents pictorial evidence of a tissue section acting as negative control for Figure 7 and represents female skin that is not stained at all by the digoxygenin-linked Y-chromosome probe.

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Figure 9 represents pictorial evidence of a lower portion of an induced follicle stained positively following in situ hybridisation with a Y-chromosome-specific DNA probe, realised via a green fluorophore marker.

10   Figure 10 represents pictorial evidence of a tissue section acting as a positive control for Figure 9.

Figure 11 represents pictorial evidence of a high power magnification view of the side of a long term [24 days] graft.

15

Figure 12 represents pictorial evidence of dermal sheath cell capability to differentiate into different mesenchymal cells.

(A)   Long term cultured (over a year) human dermal sheath cells.

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(B)   Dermal sheath cells appearing to fuse in myoblast (muscle-like) fashion.

(C)   Myotube-like structures in dermal sheath cell cultures.

25   (D)   Adipocyte (fat producing) cells.

(E)   Chondrocyte (cartilage-type) cells.

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(F)   Mineral producing bone precursor cells - Von Kossa stained.

(G)   Dermal sheath cells labelled immunohistochemically for alpha- smooth

5   muscle actin.

(H)   Human dermal sheath cells positively stained for smooth muscle myosin.

(I)   Dermal sheath cells labelled positively for desmin.

10   Figure 13 represents pictorial evidence of skin at the margin of a wound and in

which dermal sheath cells have surrounded an isolated follicle in the undamaged

tissue away from the main group of labelled cells remote in undamaged tissue.

Experimental Approach

15

Tissue isolation

A small patch of male scalp skin (about 1.5 cm^2) was coarsely shaven, leaving

some fibre still exposed to allow for subsequent plucking. The area was wiped

20   with an antiseptic solution and injected locally with lignocaine plus adrenaline

anaesthesia, before taking a 6 mm diameter punch biopsy at an angle

appropriate to follicle orientation. The most proximal tips (under 1/5th of

length) of the exposed follicles were amputated under a dissecting microscope

(Zeiss) from the inverted biopsy, and transferred to individual drops of minimal

25   essential medium (Gibco) at 4°C. After plucking the hair fibres from the

transected follicles, the biopsy was returned to its original scalp skin site and

left to heal. This initial procedure lasted about 20-25 mins. Refer to Figure 2

(a, a1, b, b1 and c) which represents a diagrammatic representation of

procedures.

The outermost end bulb dermal layers were inverted to allow the epidermal
matrix (including undifferentiated tissue) to be scraped away and discarded
(Fig. 2d). Dermal papillae, isolated by basal stalk severance (Fig. 2e), were
pooled in fresh medium (Fig. 2h). The thin external covering of connective
tissue was then teased from the pieces of sheath dermis before they were
similarly pooled in fresh medium. (Fig. 2g and i). Figure 3 represents pictorial
evidence of isolated dermal papilla (P) and sheath (S) tissue microdissected
from male scalp hair follicle end bulbs as shown in Figure 2e marked by a star
(*).

Implantations

These operations were so minimally invasive as to be practically imperceivable,
hence, no form of local anaesthetic pretreatment was deemed necessary. This
also avoided the possibility that the anaesthetic might adversely affect the tiny
quantities of vulnerable dermis that were to be implanted.

A small, shallow wound was made in the inner forearm of the female recipient
with the point of a scalpel blade, and; widened slightly using the tips of very
fine (No.5) watchmakers forceps (Fig. 2j). In the few instances when a tiny
amount of blood or fluid was exuded, it was absorbed using tiny sterile cotton
wool balls. Two sets of operations were performed.

In the first, dermal sheath tissue from twelve follicles were implanted into two
wound sites (six in each), approximately 10 hours after the end bulbs had been

removed from the biopsy. The second, involved the implantation of 11 pieces of dermal sheath into one wound site, 9 dermal papillae into a second, and 2 papillae (which stuck to the forceps and had to be re-implanted separately) into

5   a third, about 20 hours after biopsy. In all cases, the material was collected in as little fluid as possible and then transferred directly to the wound site, so that it could be rapidly inserted into the skin on the end of the forceps. The wounds were initially left untreated and uncovered. When hair fibres were seen emerging from the implanted sites (3-4 weeks later), small silicone rings with

10   rims were placed over them and secured using surgical tape - as a cautionary measure to protect against abrasion, please refer to Figure 4 which represents pictorial evidence of two hair fibres which have been produced in the immediate vicinity of the male dermal sheath-implanted female skin wound protected by a small silicone rubber collar and Figure 5 which represents pictorial evidence

15   of Figure 4 after the silicon collar (and plaster attachment) have been removed.

The first set of two wound sites were biopsied together as a single piece of elliptical skin, 77 days after sheath tissue implantation, and were fixed immediately in freshly prepared 4% paraformaldehyde at pH 7.3. The second

20   set of wounds (made 3 months after the first) were treated similarly - being removed 42 days post-operatively as two small (4 mm) punch biopsies (more precisely located by their positioning next to moles). Detailed external observations and photographic recordings of the male donor scalp, and recipient female arm skin sites, were made at regular intervals.

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Fluorophore-labelled Y-chromosome probe [Imagenetics]

The spectrum green fluorophore-labelled enumerator probe (Imagenetics),

consisted of chromosome-specific sequences from highly repeated human

satellite DNAs. The target DNA in the tissue sections was denatured in 70%

formamide / 2x SSC at 70 °C for 10 mins. Meanwhile, the probe mixture was

5   prepared to contain: 7ul SpectrumCEP hybridisation buffer (dextran sulphate,

formamide, SSC, pH 7.0), 1ul SpectrumCEP probe (fluorophore-labelled

enumerator probe and blocking DNA in Tris-EDTA buffer) and 2ul of 5x

blocking solution (as detailed above), were centrifuged (1-3 secs), heated for 5

mins in a 75°C water bath and then placed on ice. The denatured slides were

10   washed in 70%, 85% and 100% ethanol (1 min in each) and then air dried.

Each slide, heated to 45oC, received 10 ul of probe mix and then a silanised

coverslip which was sealed at the edges prior to the slides incubation in a humid

box at 42°C for 18 hours. Following hybridisation and coverslip removal, the

slides were washed for: 3x 10 mins in 50% formamide / 2x SSC; 10 mins in 2x

15   SSC, and 5 mins in 2x SSC / 0.1% NP-40, all containing Denhardts solution, 50

ug/ml sonicated salmon sperm DNA,1% milk powder and 0.1% Tween-20 and

all at 45oC. The slides were allowed to air dry in the dark, and then 10 ul of

propidium iodide counterstain (Imagenetics) and a coverslip, added to each.

20   __Digoxigenin-labelled Y-chromosome probe [Boehringer Mannheim]__

Each slide received 20 ul of the hybridisation mixture, consisting of: 10 ul

formamide [50% of final volume]; 5 ul 4X hybridisation solution; 2.5 ul probe

[50 ng]; 2.5 ul 8X blocking solutions. The mixture was covered by a silanised

25   glass coverslip, sealed and then denatured for 5->10 mins at 72°C on a pre-

warmed glass plate in the oven, before incubation in a moist chamber at 37°C

overnight. The slides were washed for 3X 5 mins in 2X SSC, prior to 30 mins

in 50 ml TBS containing 1X blocking solution (as above) and 1% Boehringer

kit blocker reagent - both also at 37°C. To promote detection, the slides were
transferred to 50 ml TBS and 50 ul anti-digoxigenin alkaline phosphatase
conjugate [200 ug/ml] containing 1% kit blocker reagent for 30 mins at 37°C,

5   and then they were washed for 3X 10 mins in 0.2% Tween 20 in TBS at room
temperature. Immediately before use, 4.5 ul of NBT, 3.5 ul of X-phosphate and
0.24 mg of levamisole (Sigma) was added to 1ml of Tris / NaCl / MgCl2 buffer.
Appropriate volumes for the number and size of the sections were added and
the slides incubated at room temperature in a humidified box covered in foil

10   until the dark blue/purple colour developed. To stop the reaction, the slides
were rinsed for 5 mins at room temperature in 10 mM Tris-Cl / 1 mM Na2
EDTA, pH 8.0.

Sections to be counter stained with propidium iodide were incubated for 5 mins

15   at room temperature in the dark in 50 ml TBS + 5 ul propidium iodide [1mg
/ml], or a similar concentration of acriflavine yellow, washed for 2-3 mins under
running water, and then allowed to air dry in the dark. Finally, the sections
were mounted in 20 ul of anti-fading solution under a glass coverslip, which
was sealed at the edges with nail varnish.

20

Wound Contraction and Wound Closure Experiments

Collagen gel contraction experiments

25   Specific populations of adult human cells were isolated by precise
microdissection of the appropriate tissues (wastage from prior operations on 25-
45 year old male and female donors), which were then used to initiate cell
cultures by explant outgrowth. A similar approach was also employed to

22

establish the different ages of rodent cells (as detailed below). Primary cultures (human and rat) were subpassaged about 2 to 3 weeks after their initiation, and all of the experiments were performed with either second or third passage cells

5   some 2 to 4 weeks subsequently.

Type 1 collagen solution was prepared from adult rat tail tendons (20, 21). In brief, 1g of U.V./ethanol-sterilised tail tendon was stirred in 300ml of sterile 0.5M acetic acid for 48 hours at 4°C. The resulting solution was then filtered

10   through several layers of sterile gauze, centrifuged at 2500g for 3 hours, and dialysed for 24 hours against distilled water before undergoing further centrifugation. The collagen gel (which sets at 37°C) was prepared by mixing the collagen solution with one tenth of it's volume of 10X concentrated MEM and approximately half of this volume of 4.4% $NaHCO_3$ to give a final pH of

15   7.3. Each population was seeded at the same density of 10^5 cells per ml of collagen gel, 1.5ml of which was placed into each 35mm petri dish at 37°C (95% air / 5% CO_2) to set and then covered with about 0.5ml of MEM (considered to be time = 0). The diameter of 20-24 separate collagen gels were recorded for each cell type, every 4-6 hours for a period of 5 days. Further

20   medium was added in small increments as the gels shrank.

We conducted further experiments to investigate the "knitting together" of cut edges of skin. Standardised cuts were made in small oblong pieces of human skin, either vertically across three quarters of their length, or horizontally right

25   through them, just below the epidermis. The cut surfaces were then pressed back together with either skin fibroblasts, dermal papilla, dermal sheath or smooth muscle cells (that had been amassed into "tacky" lumps of viable cells with a rubber scraper) and subsequently sandwiched between. Very confluent

petri dishes of cells were far superior sources to those that were low in numbers, since they produced stickier accumulations of cells. All of the pieces of skin were cultured in a non-liquid environment, that is on a permanently damp

5   surface in a humid atmosphere 37°C, but never floating on, or submerged in, medium.

Storage of Dermal Sheath Tissue

10   Cold temperature storage of dermal sheath tissue/cells; additionally their subjection to adverse conditions to highlight stem cell-type characteristics - including capacity for preferential survival.

Human skin samples (as detailed directly above) were cleaned and appropriately

15   microdissected to provide: (a) 3mm^2 portions of whole skin; (b) isolated hair follicles; (c) fragments of glassy membrane sandwiched between thin layers of sheath dermis and ORS epidermis, and (d) primary cultures of dermal sheath cells (prepared as above). Each of these four levels of tissue complexity were then subjected to six different forms of adverse conditions (each repeated with

20   and without serum, and/or, glucose and glycerol): (i) prolonged cold temperature storage at 4°C; (ii) repeated freeze/thaw cycles at -20°C; (iii) repeated freeze/thaw storage at -80°C in DMSO;

RESULTS

25

Sheath implants

All of the sites that had been implanted with dermal sheath tissue healed rapidly

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and in a manner that seemed typical of any superficial skin lesion. Fine narrow scabs formed as the site dried and then were lost over the next few days to leave a very faint wound, which was almost imperceivable by about the 10th day.

5   There was no external sign of any inflammatory reaction in or around the wounds, nor any physical perception of the site. The tip of a fibre that was darker and disproportionately sturdier for its length than any of the arm skins local vellus hairs, was first noticed on the 24th day after the dermal sheath had been introduced. On the 33rd day post-implantation, a second much finer and

10   unpigmented fibre was seen to have emerged just to the side of the first. A very light peppering of pigmented material was also visible below the surface of the skin, in the immediate vicinity of the healed sites. In addition, a dark line of material could be seen underneath the skin directly behind the base of the larger fibre (refer to Figures 4 and 5). This almost certainly represented a continuation

15   of the exposed length of hair, and indicated that the follicle producing it was shallowly embedded and at an unusual angle and orientation relative to the local follicles. Both fibres increased in mass and length over the next few weeks, but this was more pronounced in the pigmented fibre which became more obviously stouter and thus morphologically distinct from the local hairs (refer to Figures

20   4 and 5). The finer white fibre was covered by a thin layer (or sac) of dried cells, but otherwise, was quite similar in stature and general appearance to the neighbouring non-induced hairs. Twenty one days after the second set of operations (initiated three months after the first) a fibre (again darker and sturdier than the local hairs) was seen at the sheath-implanted site. Over a

25   further similar time span of three weeks, this solidly pigmented hair grew thicker and became more curved. The site was biopsied on day 42.

Histological examination of the sheath-implanted sites confirmed that the two

25

larger follicles which had produced terminal-type fibres externally, had all of the characteristic components. For instance, large oval (Alcian blue-positive) dermal papillae (Fig. 6, legend P) were overlaid by a pigmented epidermal

5   matrix, and follicle-typical concentric tissue layers could also be clearly seen in transverse sections. However, these follicles were quite different from the local vellus population in terms of their: larger size; shallow depth of growth within the skin, and unusual angle of orientation parallel to the skin surface. Such independent and contrasting features strongly suggest that the larger

10   appendages were induced.

Notably, none of the transplanted material was transplanted into an immunoprotected site.

15   Further smaller follicles were also noted in random positions and arrangements in and around the post-experimental wound sites, and while they too may have been newly formed, their situation could not be interpreted on the basis of the morphological criteria alone.

20   <u>Evidence in support of immunoprivilege as illustrated by in situ hybridisation</u>

Both positive (refer to Figure 7 which represents pictorial evidence of a lower portion of an induced follicle which can be seen to stain positively following in-

25   situ hybridisation with a Y-chromosome-specific DNA probe, realised via digoxygenin label) and negative (refer to Figure 8 which represents pictorial evidence of a tissue section acting as a negative control for Figure 7, and represents female skin that is not stained at all by the digoxygenin-linked Y-

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chromosome probe) controls stained appropriately to confirm the validity of the protocols basic methodology.

5   In the first set of experimental tissue sections, both of the Y-chromosome-specific DNA probes recognised some of the smaller follicles in the wound sites, as well as the more predictably induced larger ones. Only the lowermost regions of the smaller follicles, in fact, little more than the end bulb regions, repeatedly stained positively with the probes (compare Figures 7 and 8), as
10   visualised by either the digoxygenin or the Spectrum green fluorophore to indicate the cells of male origin. Unfortunately, the morphological resolution of the tissue was not adequate to interpret the probes distribution at the level of individual cells, or even tissue layers. Nevertheless, that both the fluorophore, (refer to Figure 9 compared to Figure 10) and digoxygenin - (Figures 7
15   compared to 8) labelled probe recognised almost identical regions of the follicles tissue as positive, was considered to reinforce the results.

Experimental Evidence in support of the ability of dermal sheath cells to provide long term replacement skin dermis
20

Dermal sheath cells were recombined with epidermal cells from hair follicles and grafted, inside a chamber that separated the graft from the surrounding skin cells, onto an animal.

25   The dermal sheath cells formed a very good dermis with uniform cell density and no sign of abnormal collagen formation. They also interacted with the epidermis to produce a thick epidermal covering. A complete and normal basement membrane was formed between dermal sheath and epidermis. Where

the chamber surrounding the graft has been removed, the white block cell

infiltrate that has built up outside the graft does not appear to enter the new skin

site. Refer to Figure 11 which represents a high power magnification view of

5   the side of a long term [24 days] graft. The line of dark dense white blood cell

infiltrate on the left, has not encroached into the graft site. In the dermis,

collagen bundles are structured, dermal cells are regularly distributed and a

complete and normal basement membrane is obvious.

10   **Experimental Evidence in support of dermal sheath cell stem cell potential**

Figure 12 (A-I) represent pictorial evidence of dermal sheath cells capability to

differentiate into different mesenchymal cells and hence their stem cell

potential. It can be seen that these cells can differentiate into myotubes,

15   adipocytes, chondrocytes and mineral producing bone cells. Further surprising

evidence includes hair follicle tissue, obtained from individuals in the 95-105

age range, was found to be viable and capable of acting as a productive source

for cell culture initiation. This data supports the hypothesis of the capability of

stem cells to differentiate and reproduce remains constant during lifetime (22).

20   Additionally repeated freezing and thawing of primary dermal sheath cells and

subsequent cloning did not alter their potential to exhibit at least four different

phenotypes despite their prior exposure to adverse conditions.

Experimental Evidence in Support of Follicle dermal sheath lineage
25   **potential**

Muscle myotubes

Subpopulations of small spindle-shaped cells were observed both singularly and in various states of fusion (as can also be commonly seen in routinely prepared cultures), some forming long branching, multinucleate myotube-like structures.

A proportion of these cells strained positively with myosin, desmin and/or alpha-smooth muscle actin monoclonal antibodies. [There have even been an odd occasion in the past when we have observed spontaneous rhythmic beating, i.e. contractions, of long aggregations of such muscle precursor-type cells in our petri dishes].

Adipocytes

These cells were identified by their distinctive multivesiculate appearance and the fact that the material contained within their vesicles was stained red by Sudan IV, and thus shown to be saturated neutral lipid.

Chondrocytes

Seen as accumulations of rounded cells with pericellular pH 1.0 Alcian Blue positive material which would be chondriotin and keratan sulphate proteoglycans, and lacunae between many of the cells - {interestingly similar cell behaviour is observed when rat dermal sheath cells are mixed with microdissected ear cartlidge in vitro}. This also seems likely to be related to our observations in vivo, when implanted dermal sheath cells appear to simulate hyperplasia in the normally inactive ear cartilage.

29

Mineral producing Bone Cells

These cells were identified by their formation of aggregates in which the matrix
appeared mineralised and stained positively for calcium phosphates, after being
treated by the von Kossa method.

Further distinctive cell types have also been observed in our dermal sheath cell
cultures (including interesting dendritic populations) but as yet these remain
inaccurately defined.

Experimental Evidence in support of dermal sheath cells as substitutes for fibroblasts in skin wounding

Fluorescent dye (DiI) labelled dermal sheath cells and fibroblasts were
implanted into skin wounds in a collagen gel, dermal sheath cells survived
comparably to skin cells over 10 days and were observed to penetrate further
into host skin. Dermal sheath cells were also shown to be capable of migration
and incorporating themselves into normal skin away from the wound itself
(refer to Fig. 13 which represents pictorial evidence of skin at the margin of a
wound and in which dermal sheath cells have surrounded an isolated follicle
remote in undamaged tissue).

Evidence in support of wound contracture

Wound contracture is a very important part of the process by which skin heals,
and it is thought that a transient population of cells called myofibroblasts, effect
this closing of the damaged site by contraction. The exact source of the

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myofibroblasts has until now, not been identified, but they are characterised by
their expression of alpha-smooth muscle actin.

5   We conducted studies which compared the abilities of follicular and non-
follicular dermal cells from three different ages of rat, and from different skin
sites in adult humans, to contract collagen gel lattices. The adult human cell
types involved were derived from skin fibroblasts from four different body sites;
specifically smooth muscle cells; scalp follicle dermal papilla and, lower, mid

10 and upper follicle dermal sheath cells. The rodent cells were derived from
newborn, 14 day, and adult animals skin fibroblasts from four different body
sites, [ear, mystacial, dorsal, footpad]; aortic smooth muscle cells, and vibrissa
follicle dermal papilla and sheath cells.

15 Results showed that all of the follicle-derived dermal cell types were able to
contract gels to a much greater extent than (roughly double) either the smooth
muscle cells, or any of the body regions of skin fibroblasts. The contractile
capabilities of the adult rat vibrissa-derived dermal cells, was similar to that of
the newborn rat skin fibroblasts and smooth muscle cells. Although, in any one

20 age group, the follicle-derived cells were always more contractile than either
skin fibroblasts or smooth muscle cells (which behaved quite similarly).

Of the four cell types investigated, the dermal sheath cells were by far the most
able promoters of the "knitting together" process. They alone formed stable

25 joins between two damaged edges of skin portion, whether the cut had been
made vertically, or horizontally. Occasionally, they appeared to have actually
sealed the cut surfaces together, since no gaps were evident externally as the
overlying epidermis was continuous. The dermal papilla cells, while a lot less

31

effective than the dermal sheath (perhaps a third as good), were better than controls of no cells at all. The smooth muscle cells capabilities to effect reassociation of the tissue seemed on a par with not having introduced any cells

5   into the wounds, but the skin fibroblasts actually appeared to hinder any "sticking" at all.

Although conducted in vitro, this work supports the proposition that follicle-derived dermal cells may promote wound healing processes in vivo.

10

Storage of Dermal Sheath Tissue

Our investigations have shown that dermal sheath tissue and/ or cells derived therefrom can be stored long term at low temperatures and yet still, when

15   subjected to appropriate conditions, grow. This clearly has important implications in the storage of wound healing therapeutics, and specifically, the storage of grafts or "living skins" made therefrom.

Moreover, our investigations have also shown that the dermal sheath cells can

20   persist for a long time in culture under extreme stress. This is also of important implications for wound healing therapeutics derived from this tissue type and tends to simply that the tissue type is suitably robust, and displays stem cell characteristic durability and viability.

25   In short, not only does dermal sheath tissue and/or cells derived therefrom have all the advantageous properties that one might hope to find in a wound healing tissue type but it also has properties that facilitate the use of the tissue in terms of manufacturing and long term storage.

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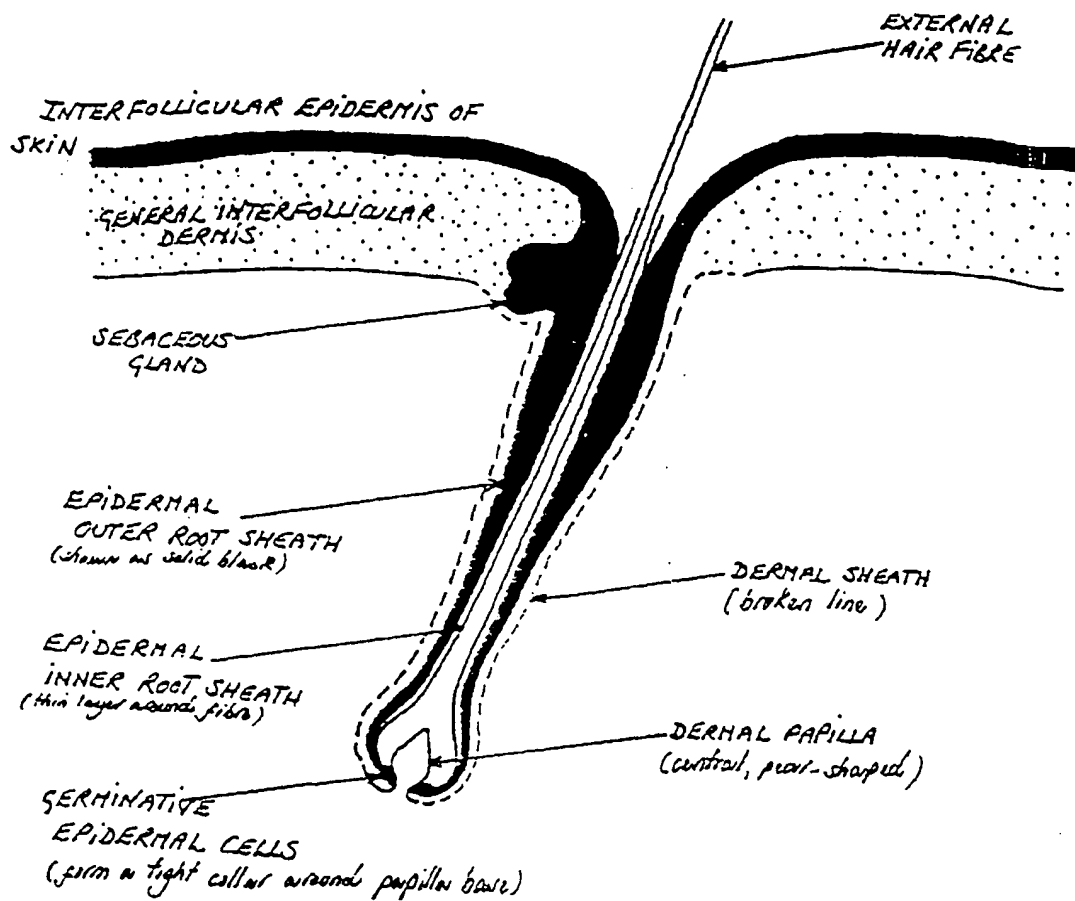
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CLAIMS

1.   A wound healing system comprising dermal sheath tissue.

2.   A wound healing system according to Claim 1 wherein said dermal sheath tissue is derived from a selected portion of a hair follicle.

3.   A wound healing system according to Claim 2 wherein said selected portion is the lower third of a hair follicle.

4.   A wound healing system according to a previous claim wherein said dermal sheath tissue is derived from a ring of cells about a hair follicle.

5.   A wound healing system according to Claim 4 wherein said ring of cells is an inner ring of cells with respect to the dermal or mesenchymal region of the hair follicle.

6.   A wound healing system according to any proceeding Claim wherein said dermal sheath tissue is characterised in that it stains positive for alpha smooth muscle actin.

7.   A wound healing system according to any proceeding Claim wherein said dermal sheath tissue is provided, or combined, with at least one other tissue or cell type, from a hair follicle.

8.   A wound healing system according to Claim 7 wherein said other tissue or cell type comprises dermal papillae or cells derived therefrom.

9.   A wound healing system according to a preceding Claim
wherein said system comprises a suitable matrix material.

10.   A wound healing system according to Claim 9 wherein said material
comprises native collagen, or collagenous gels or lattices constructed from
reconstituted collagen or highly complex mixtures of reconstituted collagen.

11.   A wound healing system according to Claims 9 or 10 wherein said
system further comprises a web of material onto which said matrix is
applied, such that said matrix including said dermal sheath tissue is
incorporated or embedded in, or associated with or attached to said web.

12.   A wound healing system according to Claim 11 wherein said system
comprises a surgical dressing.

13.   A wound healing system according to Claims 1 to 11 wherein
said system comprises a therapeutic composition.

14.   A wound healing system according to Claims 12 or 13 wherein said
system further comprises at least one selected therapeutic agent.

15.   A wound healing system according to Claim 14 wherein said selected
therapeutic agent has anti-bacterial properties or anti-septic properties.

16.   A wound healing system according to Claim 14 wherein said
therapeutic agent has anaesthetic properties.

17.   A wound healing system according to Claim 13-16 wherein said therapeutic composition is adapted to be applied topically.

18.   A wound healing system according to Claims 13-16 wherein said therapeutic composition is adapted to be administered by injection.

19.   A wound healing system according to Claims 13-18 wherein said therapeutic composition is incorporated or embedded in, associated with, or attached to, a plaster or bandage.

20.   A surgical dressing comprising a suitable matrix material having incorporated and/or embedded therein, and/or associated therewith, and/or attached thereto, dermal sheath tissue and/or cells derived therefrom.

21.   A medicament comprising dermal sheath tissue.

22.   An *in vitro* wound modelling system comprising dermal sheath tissue and/or cells derived therein.

23.   A wound healing system, surgical dressing, medicament or *in vitro* wound modelling system according to any preceding claim wherein said dermal sheath tissue is derived from non-self.

Figure 1

EXTERNAL
HAIR FIBRE

INTERFOLLICULAR EPIDERMIS OF
SKIN

GENERAL INTERFOLLICULAR
DERMIS

SEBACEOUS
GLAND

EPIDERMAL
OUTER ROOT SHEATH
(shown as solid black)

EPIDERMAL
INNER ROOT SHEATH
(thin layer around fibre)

GERMINATIVE
EPIDERMAL CELLS
(form a tight collar around papilla base)

DERMAL SHEATH
(broken line)

DERMAL PAPILLA
(central, pear-shaped)

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DIAGRAMATIC REPRESENTATION OF PROCEEDURES
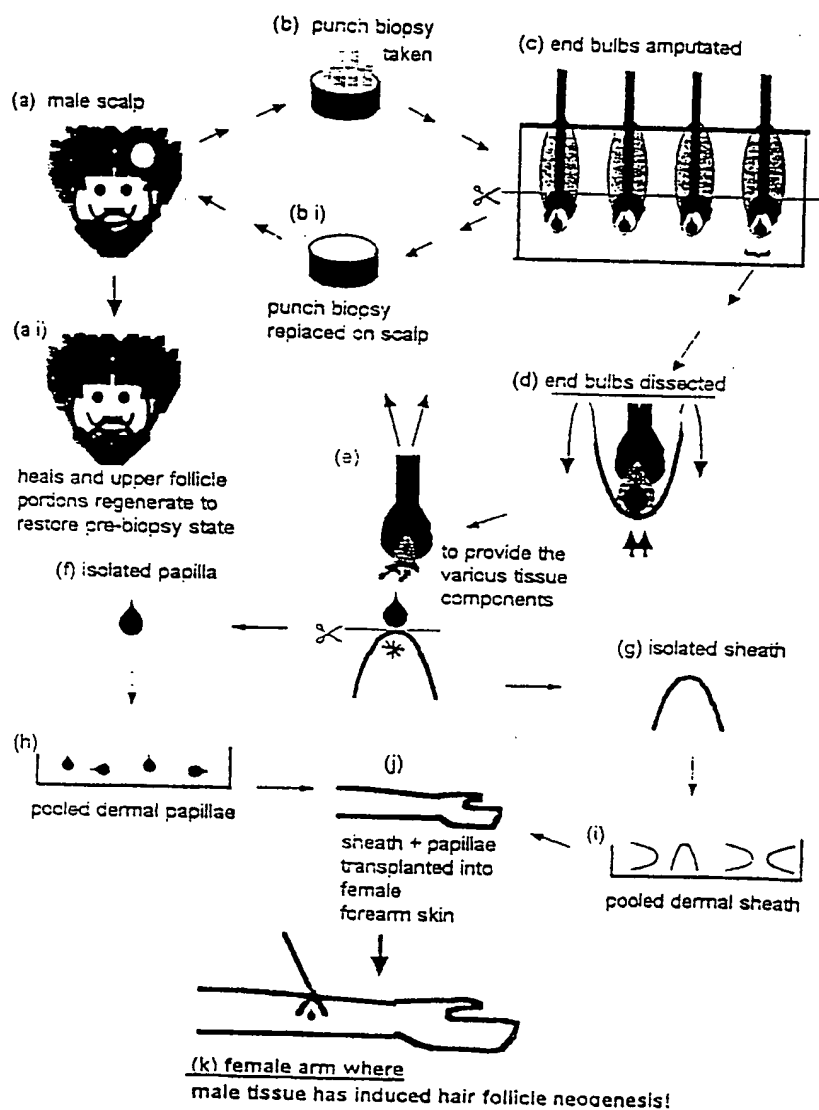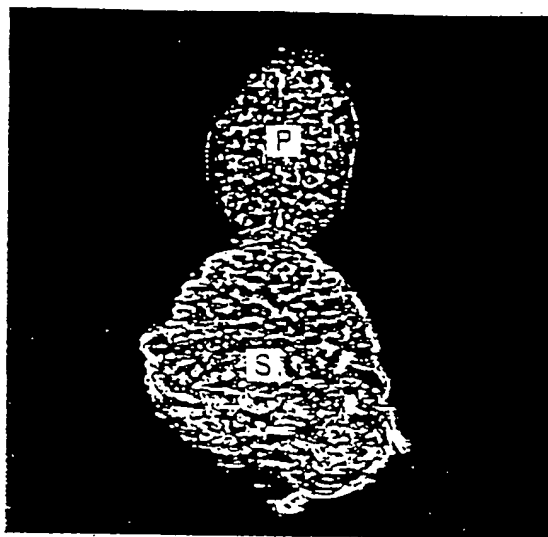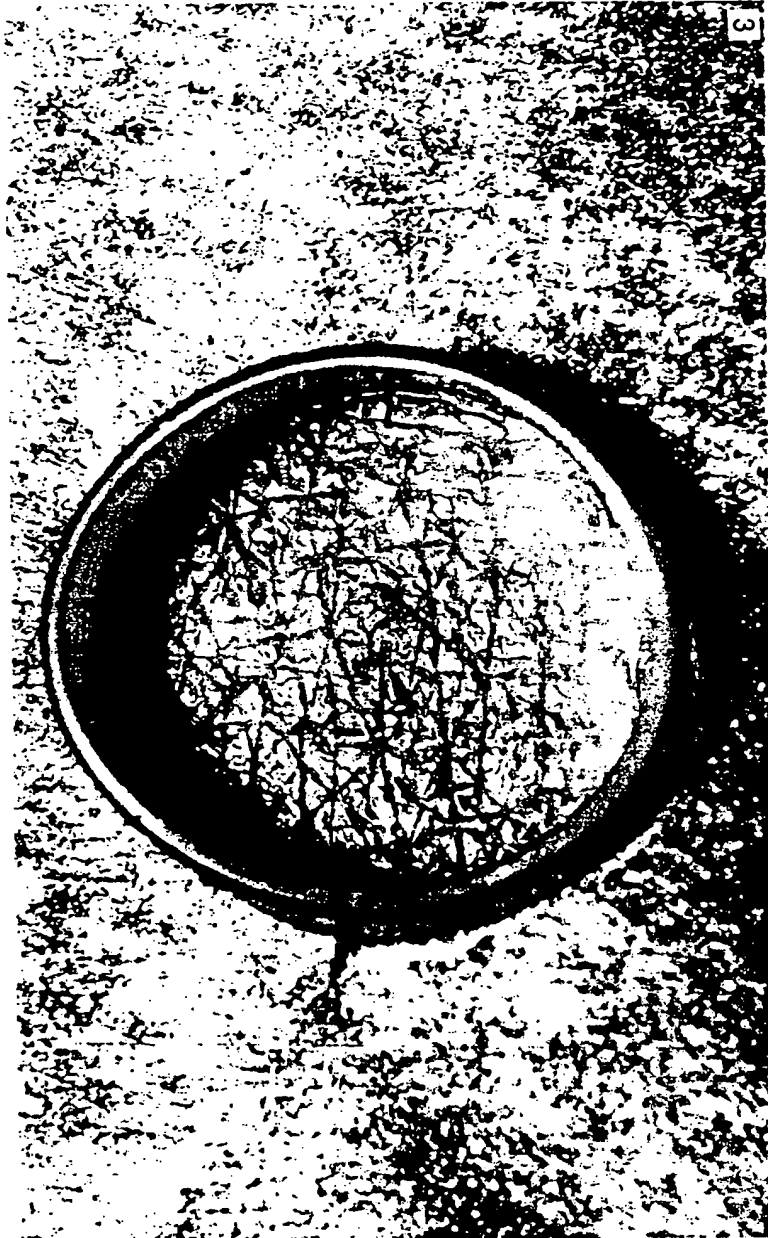
Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9
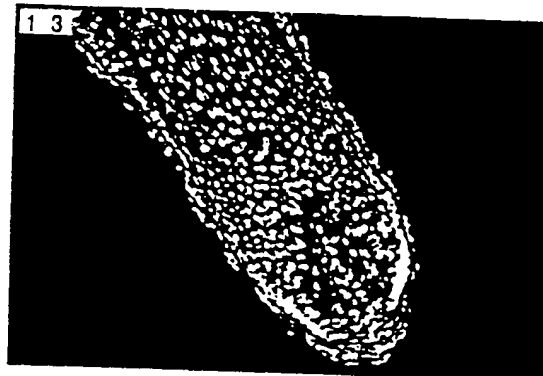
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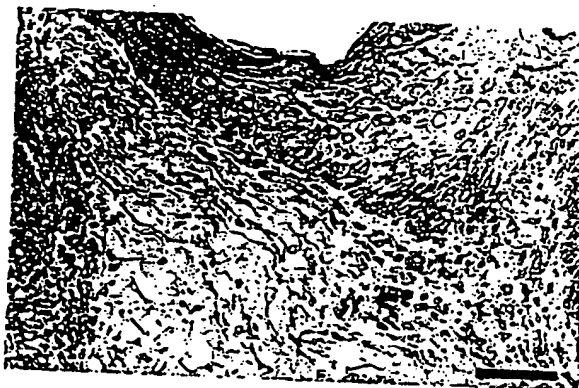
Figure 11

Figure 12A
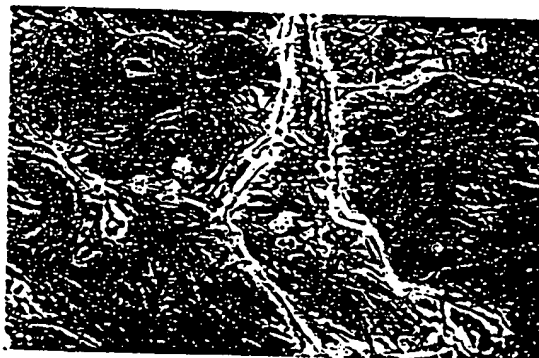
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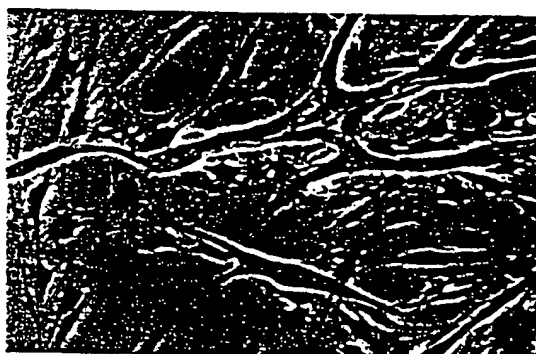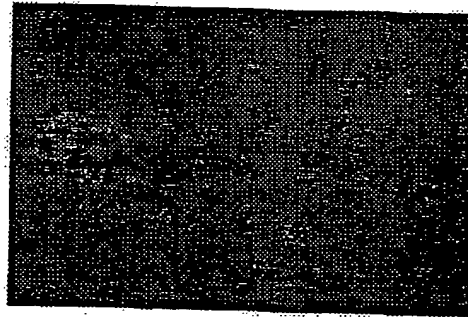
Fig 12C

Fig 12D

Fig 12E

Fig 12F
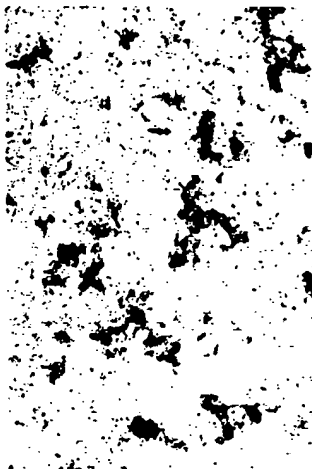
Fig 12G

Fig 12H

Fig 12I

Fig 13

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   A61L27/00   A61K35/36

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   A61L   A61K   A61F

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 01423 A (UNIV PENNSYLVANIA ;UNIV NEW YORK (US)) 12 January 1995 see page 5, line 13 - line 20; claims	1-23
X	EP 0 285 471 A (CIRD) 5 October 1988 see the whole document	1-23
X	PROUTY S M ET AL: "FIBROBLAST-DEPENDENT INDUCTION OF A MURINE SKIN LESION WITH SIMILARITY TO HUMAN COMMON BLUE NEVUS" AMERICAN JOURNAL OF PATHOLOGY, vol. 148, no. 6, June 1996, pages 1871-1885, XP000676398 see abstract	1-23
P,X	WO 97 25995 A (JOHNSON & JOHNSON CONSUMER) 24 July 1997 see claims; examples	1-23

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[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 August 1998	13/08/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	ESPINOSA, M

Form PCT/ISA/210 (second sheet) (July 1992)

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 236 014 A (UNIV DUNDEE) 9 September 1987 see claims	1-23
A	EP 0 405 656 A (UNIV DUNDEE) 2 January 1991 see claims	1-23
A	SCANDURRO A B ET AL: "IMMORTALIZED RAT WHISKER DERMAL PAPILLA CELLS COOPERATE WITH MOUSE IMMATURE HAIR FOLLICLE BUDS TO ACTIVATE TYPE IV PROCOLLAGENASES IN COLLAGEN MATRIX COCULTURE: CORRELATION WITH ABILITY TO PROMOTE HAIR FOLLICLE DEVELOPMENT IN NUDE MOUSE GRAFTS" JOURNAL OF INVESTIGATIVE DERMATOLOGY, vol. 105, no. 1, July 1995, pages 177-183, XP000196475 see abstract	1-23
A	WO 93 25660 A (BRIGHAM & WOMENS HOSPITAL) 23 December 1993 see claims; examples 1-3	1-23
A	WO 83 01384 A (MASSACHUSETTS INST TECHNOLOGY) 28 April 1983 see claims	1-23

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INTERNATIONAL SEARCH REPORT

Information on patent family members

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